Clinical listonellosis in meagre (*Argyrosomus regius*) from recirculated aquaculture system in Turkey

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Received December 4, 2017
Accepted June 27, 2018

Abstract

Vibriosis caused by *Listonella anguillarum* was reported in several fish species from both fresh and saltwater conditions. This pathogen causes disease in rainbow trout, sea bass, and sea bream in Turkey, however, it has not been reported from meagre (*Argyrosomus regius*) before. Great loss of meagre was observed in the Recirculated Aquaculture System at the Faculty of Fisheries of Izmir Katip Celebi University, which had been transferred from a commercial hatchery for a nutrition experiment. Clinical signs of vibriosis were observed in infected fish, i.e. haemorrhage in the anal area and pectoral fins, mostly as tail ulcers. Petechial haemorrhages in the muscle, liver, peritoneal membranes and pyloric caeca were determined by necropsy. A Gram-negative, rod-shaped, motile bacterium was isolated, showing a positive reaction to oxidase, catalase and gelatin tests, and being sensitive to O/129. Biochemical identification tests and PCR amplifications identified the bacterium as *Listonella anguillarum.* In slide agglutination test with anti *L. anguillarum* O1 (ATCC43305) serum, all isolates were positive. The isolated bacteria was resistant to oxytetracycline, sensitive to enrofloxacin, flumequine, phosphomycin, furozulidone, kanamycin and oxolinic acid. In this study, the isolated bacteria from meagre were determined as *Listonella anguillarum* O1 with biochemical, moleculer identification and agglutination tests.

Listonella anguillarum, fish disease, isolation

Vibriosis that has been found in more than 50 fresh and saltwater fishes is one of the most prevalent fish diseases caused by bacteria belonging to the genus *Vibrio*. The first known vibriosis outbreak was reported in 1893 in eels as *Bacterium anguillarum* (Canestrini 1907). In 1909, Bergman proposed the name of *Vibrio anguillarum* but in 1985, Mac Donell and Colwell reclassified the pathogen into the new genus, *Listonella*. Currently, both nomenclatures are present in the literature (Hickey and Lee 2017).

Listonellosis, also known as salt-water furunculosis (Rucker 1963), boil disease (Kubota and Takakuwa 1963) and ulcer disease (Bagge and Bagge 1956), is caused by a Gram-negative, polar flagellated, comma-shaped rod bacterium *Listonella (Vibrio) anguillarum* from the *Vibrionaceae* family (Actis et al. 1999). It grows on rich media containing 1.5–2.0% sodium chloride (NaCl) between 25–30°C temperatures and forms cream-coloured, round-shaped colonies (Frans et al. 2011). Chemical stress such as water quality, diet composition and pollution, biological stress like population density and the presence of other macro- or micro-organisms, and physical stress due to temperature and overcrowding are important factors that cause outbreaks (Thune et al. 1993; Frans et al. 2011). In addition, the virulence of the *L. anguillarum* strains influences the onset of disease outbreaks (Actis et al. 1999). It has been postulated that in acute epizootics, infected fish dies rapidly without showing any clinical signs (Actis et al. 1999; Toranzo et al. 2005; Austin and Austin 2007; Frans et al. 2011).

In Turkey, *L. anguillarum* was reported in cultured sea bass (Çağırgan 1993) and rainbow trout (*Oncorhynchus mykiss*) (Tanrıkul 2007). Currently, additional isolates were

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described from different fish farms and species as a result of the expanding aquaculture industry. Meagre (*Argyrosomus regius*) farming started in Turkey in 2007 and now it is one of the alternative species that have increased production numbers because of its favourable biological characteristics such as being resistant to environmental conditions, having good feed conversion, fast growth, and high flesh quality.

The objective of this study was to determine and to identify the biochemical characteristics of *Listonella anguillarium* serogroup O1 from meagre. This pathogen has been known for some time but has not been reported previously in Turkey.

Materials and Methods

Fish

The outbreak was observed in the Recirculated Aquaculture System at Faculty of Fisheries of the Izmir Katip Celebi University. The fish were supplied to the university from a commercial hatchery for a nutrition experiment. The water source of the system was brackish (3.9‰) water and the temperature was 16 ±2 °C when high mortality was observed.

Bacterial strains

Isolation and identification of bacteria

A total of 25 moribund fish of 20 g were examined by bacteriological investigations. The isolates from the kidneys of diseased fish were streaked on tryptone soy agar (TSA, Oxoid, UK) including 1.5% NaCl and incubated at 21 °C for 48 h. After primer isolation, the representative colonies were passaged onto thiosulphate-citrate-bile salts-sucrose agar (TCBS, Oxoid, UK). The purified colonies were cultivated on TSA including 1.5% NaCl for biochemical tests. Gram staining and oxidase test were carried out according to standard procedures. The motility of the bacteria was detected by the hanging-drop technique. Oxidative and fermentative degradation of glucose and gas production were tested on O/F Medium (Difco, USA). Resistance to the 2,4-diamino-6,7-diisopropylpteridine O/129 vibriostatic agent was tested with discs (Oxoid, UK) on TSA (Oxoid, UK) including 1.5% NaCl. Biochemical tests for further information were carried out with API 20E (BioMerieux S. A., France) (Tanrikul et al. 2005) which was carried out at 21°C for 48 h. Sterile 1.5% saline was used for inoculation of the bacterium to API 20E strips.

Preparation of antisera and slide agglutination test

Slide agglutination test was performed according to Toranzo et al. (1983). Rabbit serum was used against all isolated *L. anguillarım* O1 strains (ATCC 43305). Bacterial growth occurred on brain heart infusion agar (BHIA, Oxoid, UK) plates at 25 °C for 24–28 h. Bacterial strains were resuspended in PBS (phosphate buffered saline solution) and reached the concentration of McFarland Standard No. 3 for use as antigens in the slide agglutination tests.

Molecular identification of strains

Molecular identification of the bacteria was conducted. The 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) in order to confirm that the bacteria were *Listonella anguillarum*. The pathogen was obtained from the samples that were isolated from infected meagre during the outbreak. EurX GeneMATRIX Tissue Bacteria Isolation Kit (EURx Ltd., Poland) was used for DNA isolation. Then with use of Nanodrop 2000 (Thermo Scientific, USA), density and quality of the isolates were determined. The 27F and 1492R primers were used for PCR amplifications. Band screening of the PCR products was observed in the gel electrophoresis. Amplified products of template DNA were sent to the Stab Vida direct sequencing service (Spain) with the ABI 3730 XL DNA Analyzer for sequence determination. Then the sequences were checked against the BLASTN 2.6.1. database.

Antimicrobial sensitivity test

Antibiotic susceptibility of the pathogen was tested with 8 antibiotics on Mueller-Hilton agar (MHA, Merck, Germany) using the Kirby-Bauer disc diffusion technique (Stokes et al. 1993). A loopful of 24 h culture was placed in the center of a Petri dish with media and spread with a dry swab. Test discs of enrofloxacin, florfenicol, flumequine, phosphomycin, furozulidone, kanamycin, oxolinic acid, oxytetracycline and sulphamethoxazole/trimethoprim (Oxoid, UK) were placed on the dishes and incubated at 21 °C for 24 h. The disk diffusion zone diameters were measured (mm) and compared with the interpretive criterias of National Commite for Clinical Laboratory Standarts (NCCLS, 1993, 1994).

Results

The affected meagre exhibited erratic swimming, dark discolouration, haemorrhage in the anal area and pectoral fins, and tail ulcers as clinical signs. High mortality was observed

and calculated as 30% during the outbreak. Necropsy showed petechial haemorrhage in the muscle, liver, peritoneal membranes and pyloric caeca from the internal sides of the fish (Plate VIII, Fig. 1).

The morphologic and biochemical properties of isolated *L. anguillarum* O1 are presented in Table 1. The isolates were found to be Gram-negative, motile, oxidase and catalase positive. According to API 20E rapid identification tests, ONPG (ortho-nitro-phenyl-galactoside), ADH (arginine dihydrolase), citrate utilization, VP (Voges Proskauer test), gelatin degradation, acid production from glucose, mannitol, inositol, sorbitol, arabinose and sucrose were found positive; LDH (lysine decarboxylase), ODC (ornithine decarboxylase), H_2S, urease, TDA (tryptophan deaminase), indole, rhamnose, melibiose and amygdalin were detected negative in all isolates from diseased fish. In slide agglutination test with anti *L. anguillarum* O1 (ATCC43305) serum, showed that all 12 isolates were positive. No other pathogen was detected from the diseased fish.

Table 1. The morphologic and biochemical properties of isolated *Listonella anguillarum*

Properties	*L. anguillarum* O1 strains
Gram stain	- (12/12)
Motility	+ (12/12)
Oxidase	+ (12/12)
Catalase	+ (12/12)
O/F	F (12/12)
O/129	+ (12/12)
ONPG	+ (12/12)
ADH	+ (12/12)
LDC	- (12/12)
ODC	- (12/12)
Citrate utilization	+ (12/12)
H_2S	- (12/12)
Urease	- (12/12)
TDA	- (12/12)
Indole	- (12/12)
VP	+ (12/12)
Gelatin	+ (12/12)
Glucose	+ (12/12)
Mannitol	+ (12/12)
Inositol	+ (12/12)
Sorbitol	+ (12/12)
Rhamnose	- (12/12)
Sucrose	+ (12/12)
Melibiose	- (12/12)
Amygdalin	- (12/12)
Arabinose	+ (12/12)

+ positive; - negative
O/F (Oxidative-fermentative medium),
O/129 (2,4-diamino-6,7-diisopropylpteridine),
ONPG (ortho-nitrophenyl-β-galactoside),
ADH (arginine dihydrolase),
LDC (lysine decarboxylase),
ODC (ornithine decarboxylase),
H_2S (H_2S production),
TDA (deaminase), VP (acetoin production)

The PCR amplification of the *L. anguillarum* gene sequence was registered in the BLASTN 2.6.1 database. It resulted in 100% nucleotide identity between the current isolate and *Listonella anguillarum* (accession number CP023208.1).

The antibiotic susceptibility results of isolated *L. anguillarum* O1 strains are presented in Table 2. The pathogen showed resistance to oxytetracycline and it was susceptible to enrofloxacin, flumequine, phosphomycin, furozulidone, kanamycin and oxolinic acid. The intermediate intensity of antibiotics were detected in florfenicol and sulphamethoxazole/trimethoprim test groups.

Infected fish were treated with enrofloxacin added to dry pellet feed (50 mg·kg^{-1} fish per day) for 7 consecutive days with 1.3% feed ratio.

Discussion

Listonella (Vibrio) anguillarum is a worldwide known bacterial pathogen causing typical haemorrhagic septicaemia in a great variety of fish species (Powell et al. 1990; Rad and Shahsanavi 2010). When fish are under stress due to overcrowding or when immunocompromised, outbreaks are usually observed caused by vibrio strains (Thune et al. 1993). Major changes in the water salinity and stress made meagre more vulnerable to fish pathogens in this vibriosis outbreak. Erratic swimming, haemorrhage in the anal and pectoral fins, dark discolouration, petechial haemorrhage in the muscle, liver, pyloric caeca, and peritoneal membranes were observed as typical clinical signs (Demircan and Candan 2006; Tanrıkul 2007).

Table 2. Antibiotic susceptibility profile of the isolated *Listonella anguillarum* O1 strains.

Antibiotic (µg/disc)	Isolate (12/12)	Antibiotic (µg/disc)	Isolate (12/12)
Enrofloxacin (5)	S (37 mm)	Kanamycin (30)	S (22 mm)
Florfenicol (30)	I (15 mm)	Oxolinic acid (30)	S (33 mm)
Flumequine (30)	S (42 mm)	Oxytetracycline (30)	R (2 mm)
Fosfomycin (50)	S (46 mm)	Sulphamethoxazole /	
Furozulidone (50)	S (39 mm)	Trimethoprim (25)	I (14 mm)

S: sensitive; R: resistant, I: intermediate

Vibrio disease was reported by Rucker in 1954 and since then it has appeared in different fish species such as yellowtail (*Seriola quinqueradiata*) (Jo et al. 1979), ayu (*Plecoglossus altivelis*) (Muroga and Egusa 1967), turbot (*Scophthalmus maximus*) (Horne et al. 1977), striped bass (*Morone saxatilis*) (Toranzo et al. 1983), cod (*Gadus morhua*) (Egidius and Andersen 1984), red sea-bream (*Pagrus major*) (Muroga and Tatani 1982), European eel (*Anguilla anguilla*) (Rodsaether et al. 1977), Japanese eel (*Anguilla japonica*) (Kitao et al. 1983), gilthead sea-bream (*Sparus aurata*) (Paperna et al. 1977), and tilapia (*Oreochromis auretus*) (Tareen 1984). In meagre (*Argyrosomus regius*) Haenen et al. (2014) reported *V. anguillarum* serotype O1 from offshore cages. In Turkey vibriosis has occurred at mariculture facilities for a long time. Especially *V. anguillarum* O1 was isolated from sea bream, sea bass and rainbow trout farms (Çağırgan 1993; Tanrıkul et al. 2005; Korun and Timur 2008).

Meagre was indicated as fast-growing, fairly fecund and longlived but it has been reported that the biology, ecology and fisheries of this species are poorly documented, especially in European waters (Quéméner 2002; Prista 2013). In recent years it has been considered as a potential candidate for culture in Turkey. Diseases caused by *L. anguillarum* were detected in both hatcheries and cages with increasing production rates. Juveniles were destroyed and adult individuals were treated with antibiotics without determining the pathogen to fight the disease. The disease has been known in hatchery and cage culture of meagre in Turkey but has not been reported before. Therefore, this study is the first declaration of *Listonella anguillarum* O1 from *Argyrosomus regius* in Turkey.

Phenotypic characteristics like Gram staining, motility, oxidase and catalase reactions of *L. anguillarum* strains were detected in accordance with Austin and Austin (2007). The API 20E rapid identification systems have been used for *L. anguillarum* strains (Tanrıkul et al. 2005; Tanrıkul 2007). According to the API 20E results the isolate was identified as *L. anguillarum* and no biochemical differences were detected on the isolated *L.anguillarum* strains. These findings are similar to Tanrikul et al (2005), Demircan and Candan (2006), Korun (2006) and Avsever (2014) for different marine species, mostly sea bass (*Dicentrarchus labrax*) as well as isolations from rainbow trout (Tanrikul 2007; Tanrikul and Gultepe 2011). Some indicators such as ADH, indole, inositol, sorbitol and arabinose vary among different studies (Table 3), but biochemical results are not the essential criteria to identify the bacteria. The results of biochemical studies showed that biochemical properties in different species do not vary with the salinity of water (Table 3).

The sensitivity of *L. anguillarum* strains to chemotherapeutics has been decreasing so far (Takahashi et al. 1976). In antimicrobial susceptibility tests, resistance to oxytetracycline was detected in the bacteria. Tanrikul (2007) reported that some *L. anguillarum* strains isolated from rainbow trout showed resistance to enrofloxacin and oxytetracycline. On the contrary, the strains isolated from aquarium catfish were determined sensitive to enrofloxacin and oxytetracycline, but resistant to penicillin (Rad and Shahsavani 2010).

Table 3. The morphologic and biochemical test results of *Listonella anguillarum* from different studies.

	Isolated *L. anguillarum* (12/12)	Tanrikul et al. 2004	Demircan and Candan 2006	Korun 2006	Avsever 2014	Tanrikul 2007	Tanrikul and Gultepe 2011
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Catalaze	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Growth in 0% NaCl	ND	+/-	ND	-	-	-	-
Growth in 7% NaCl	ND	+/-	+	-	+	-	-
Sensivity to O/129	+	+	+	+	+	+	+
O/F	F	ND	F	F	F	F	F
ONPG	+	+	+	+	+	+	+
ADH	+	+	+	+	-	+	+
LDH	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-
Citrate utilization	+	+/-	+	ND	ND	+	+
H_2S	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
TDA	-	-	-	ND	ND	-	-
Indole	-	+/-	+	+	+	-	-
VP	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Inositol	+	+/-	-	-	-	+	+
Sorbitol	+	+/-	+	+	-	+	+
Rhamnose	-	-	-	-	+	-	-
Sucrose	+	+	+	+	-	+	+
Melibiose	-	-	-	-	+	-	-
Amygladin	-	-	-	-	ND	-	-
Arabinose	+	-	+	-	+	+	+

+ positive; - negative

O/F (Oxidative-fermentative medium), O/129 (2,4-diamino-6,7-diisopropylpteridine),
ONPG (ortho-nitrophenyl-β-galactoside), ADH (arginine dihydrolase), LDC (lysine decarboxylase),
ODC (ornithine decarboxylase), H_2S (H_2S production), TDA (deaminase), VP (acetoin production),
ND (not done)

These results showed that antimicrobial susceptibility may vary depending on the strain. In addition, *L. anguillarum* which is a fish pathogen is not included in the EUCAST list, so these results could not be compared with their criteria but the zone diameters were resulted according to the NCCLS criteria..

In conclusion, there is not sufficient information about meagre infected with *L.anguillarum* in literature. It was not reported in Turkey before and limited information is reported from different countries. Haenen et al. (2014) mentioned this pathogen in different species including meagre in their review but this is the first scientific research about the microbiology and identification of this pathogen from meagre. There is limited information regarding diseases in this fish species and the findings of our study may contribute to meagre health management.

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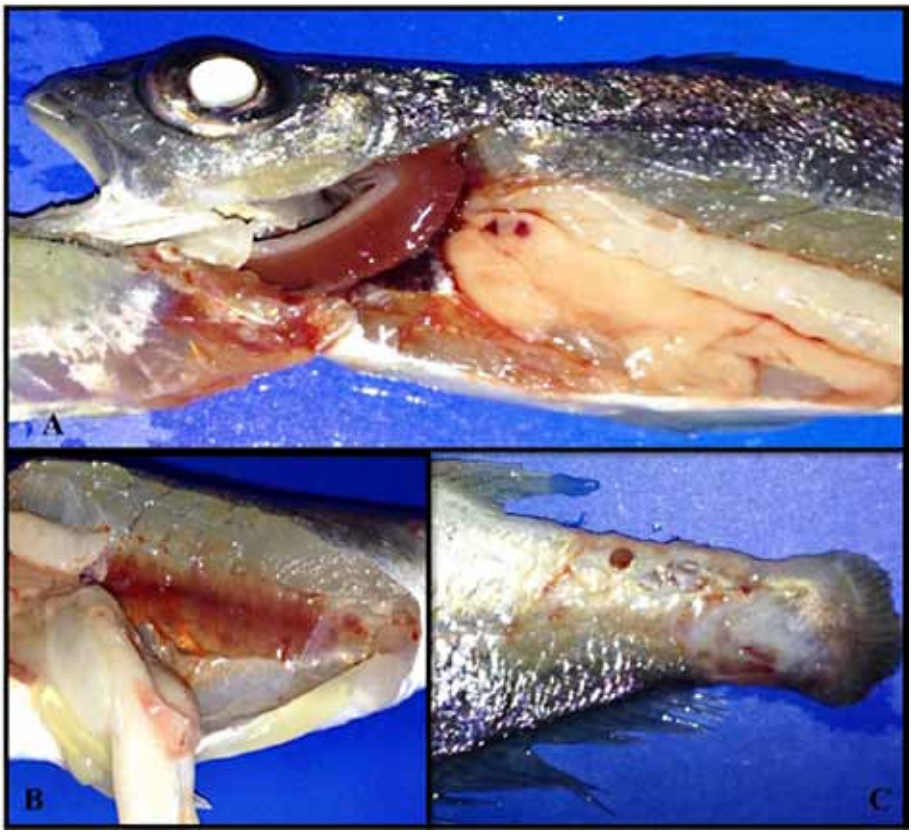

Fig. 1.A: Petechial haemorrhage in the liver; B: haemorrhage in the kidneys; C: tail ulcer